

=> d his full

(FILE 'HOME' ENTERED AT 08:12:06 ON 06 AUG 2006)

INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, ANTE, AQUALINE,
AQUASCI, BIOENG, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA, CAPLUS,
CEABA-VTB, CIN, CONFSCI, CROPB, CROPU, DDFB, DDFU, DGENE, DISSABS, DRUGB,
DRUGMONOG2, DRUGU, EMBAL, EMBASE, ...' ENTERED AT 08:12:22 ON 06 AUG 2006
SEA GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR

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986 FILE PROMT
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1788 FILE SCISEARCH
11 FILE SYNTHLINE
1158 FILE TOXCENTER
4724 FILE USPATFULL
483 FILE USPAT2
2 FILE VETB

41 FILE VETU
22 FILE WATER
871 FILE WPIDS
7 FILE WPIFV
871 FILE WPINDEX
48 FILE IPA
10 FILE NAPRALERT
506 FILE NLDB

L1 QUE GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR
BIOSYNTH?)

—
D RANK

FILE 'USPATFULL, CAPLUS, BIOSIS, MEDLINE, EMBASE, BIOTECHNO, SCISEARCH,
ESBIOBASE, PASCAL, LIFESCI' ENTERED AT 08:20:19 ON 06 AUG 2006

L2 25082 SEA GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR
BIOSYNTH?)
L3 225 SEA L2(S)(ACETYLTRANSFERAS?)
L4 134 SEA L3(S) PHOSPHAT?
L5 95 DUP REM L4 (39 DUPLICATES REMOVED)
L6 64 SEA L5(S)(SYNTHETAS? OR SYNTHAS?)
D TI L6 1-64
D L6 IBIB ABS 6 17 24 51 54 58
D IBIB ABS L6 3

Connecting via Winsock to STN

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LOGINID:ssspta1652dmr

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* * * * * * * * * * * Welcome to STN International * * * * * * * * * * *

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NEWS 2 "Ask CAS" for self-help around the clock
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NEWS 4 APR 04 STN AnaVist \$500 visualization usage credit offered
NEWS 5 MAY 10 CA/CAplus enhanced with 1900-1906 U.S. patent records
NEWS 6 MAY 11 KOREAPAT updates resume
NEWS 7 MAY 19 Derwent World Patents Index to be reloaded and enhanced
NEWS 8 MAY 30 IPC 8 Rolled-up Core codes added to CA/CAplus and
USPATFULL/USPAT2
NEWS 9 MAY 30 The F-Term thesaurus is now available in CA/CAplus
NEWS 10 JUN 02 The first reclassification of IPC codes now complete in
INPADOC
NEWS 11 JUN 26 TULSA/TULSA2 reloaded and enhanced with new search and
and display fields
NEWS 12 JUN 28 Price changes in full-text patent databases EPFULL and PCTFULL
NEWS 13 JUL 11 CHEMSAFE reloaded and enhanced
NEWS 14 JUL 14 FSTA enhanced with Japanese patents
NEWS 15 JUL 19 Coverage of Research Disclosure reinstated in DWPI

NEWS EXPRESS JUNE 30 CURRENT WINDOWS VERSION IS V8.01b, CURRENT
MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
AND CURRENT DISCOVER FILE IS DATED 26 JUNE 2006.

NEWS HOURS STN Operating Hours Plus Help Desk Availability
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NEWS IPC8 For general information regarding STN implementation of IPC 8
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=> index bioscience medicine

FILE 'DRUGMONOG' ACCESS NOT AUTHORIZED

COST IN U.S. DOLLARS

| | |
|-----------------------------|--------------------------|
| SINCE FILE
ENTRY
0.21 | TOTAL
SESSION
0.21 |
|-----------------------------|--------------------------|

FULL ESTIMATED COST

INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, ANTE, AQUALINE,
AQUASCI, BIOENG, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABAB, CAPLUS,

CEABA-VTB, CIN, CONFSCI, CROPB, CROPU, DDFB, DDFU, DGENE, DISSABS, DRUGB,
DRUGMONOG2, DRUGU, EMBAL, EMBASE, ...' ENTERED AT 08:12:22 ON 06 AUG 2006

71 FILES IN THE FILE LIST IN STNINDEX

Enter SET DETAIL ON to see search term postings or to view
search error messages that display as 0* with SET DETAIL OFF.

=> s glucosamin?(s)(synthe? or produc? or proce? or manufac? or biosynth?)

21 FILE ADISCTI
25 FILE ADISINSIGHT
30 FILE ADISNEWS
344 FILE AGRICOLA
77 FILE ANABSTR
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184 FILE AQUASCI
392 FILE BIOENG
2967 FILE BIOSIS
633 FILE BIOTECHABS

11 FILES SEARCHED...

633 FILE BIOTECHDS
1987 FILE BIOTECHNO
1095 FILE CABA
4407 FILE CAPLUS
103 FILE CEABA-VTB
37 FILE CIN

17 FILES SEARCHED...

23 FILE CONFSCI
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201 FILE DDFB
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3267 FILE DGENE

23 FILES SEARCHED...

327 FILE DISSABS
201 FILE DRUGB
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2084 FILE EMBASE
1710 FILE ESBIOBASE
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197 FILE FROSTI

33 FILES SEARCHED...

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1161 FILE GENBANK
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35 FILE KOSMET
1570 FILE LIFESCI
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45 FILES SEARCHED...

66 FILE NUTRACEUT
45 FILE OCEAN
1594 FILE PASCAL

48 FILES SEARCHED...

9 FILE PHAR
4 FILE PHARMAML
88 FILE PHIN
986 FILE PROMT

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12  FILE PROUSDDR
7  FILE RDISCLOSURE
1788 FILE SCISEARCH
11  FILE SYNTHLINE
1158 FILE TOXCENTER
60 FILES SEARCHED...
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483  FILE USPAT2
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66 FILES SEARCHED...
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871  FILE WPINDEX
68 FILES SEARCHED...
48  FILE IPA
10  FILE NAPRALERT
506  FILE NLDB

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66 FILES HAVE ONE OR MORE ANSWERS, 71 FILES SEARCHED IN STNINDEX

L1 QUE GLUCOSAMIN? (S) (SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSYNTH?)

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=> d rank
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F3      3267  DGENE
F4      2967  BIOSIS
F5      2251  MEDLINE
F6      2084  EMBASE
F7      1987  BIOTECHNO
F8      1788  SCISEARCH
F9      1710  ESBIOBASE
F10     1594  PASCAL
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F15     986   PROMT
F16     871   WPIDS
F17     871   WPINDEX
F18     804   DRUGU
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F20     633   BIOTECHABS
F21     633   BIOTECHDS
F22     546   JICST-EPLUS
F23     506   NLDB
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F25     426   DDFU
F26     392   BIOENG
F27     344   AGRICOLA
F28     327   DISSABS
F29     216   FSTA
F30     201   DDFB
F31     201   DRUGB
F32     197   FROSTI
F33     184   AQUASCI
F34     103   CEABA-VTB
F35     88    PHIN
F36     77    ANABSTR
F37     66    NUTRACEUT
F38     59    CROPU
F39     48    IPA
F40     45    OCEAN

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| F41 | 41 | VETU |
| F42 | 39 | NTIS |
| F43 | 37 | CIN |
| F44 | 35 | KOSMET |
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| F55 | 10 | NAPRALERT |
| F56 | 9 | IMSRESEARCH |
| F57 | 9 | PHAR |
| F58 | 7 | AQUALINE |
| F59 | 7 | RDISCLOSURE |
| F60 | 7 | WPIFV |
| F61 | 6 | ANTE |
| F62 | 5 | IMSDRUGNEWS |
| F63 | 4 | PHARMAML |
| F64 | 3 | CROPB |
| F65 | 2 | VETB |
| F66 | 1 | FOREGE |

=> file f1-f2, f4-f11
 COST IN U.S. DOLLARS

| | SINCE FILE ENTRY | TOTAL SESSION |
|---------------------|------------------|---------------|
| FULL ESTIMATED COST | 7.93 | 8.14 |

FILE 'USPATFULL' ENTERED AT 08:20:19 ON 06 AUG 2006
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FILE 'LIFESCI' ENTERED AT 08:20:19 ON 06 AUG 2006

```
=> s glucosamin?(s) (synthe? or produc? or proce? or manufac? or biosynth?)  
7 FILES SEARCHED...  
9 FILES SEARCHED...  
L2      25082 GLUCOSAMIN?(S) (SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSY  
NTH?)  
  
=> s l2(s) (acetyltransferas?)  
L3      225 L2(S) (ACETYLTRANSFERAS?)  
  
=> s l3(s) phosphat?  
L4      134 L3(S) PHOSPHAT?  
  
=> dup rem 14  
PROCESSING COMPLETED FOR L4  
L5      95 DUP REM L4 (39 DUPLICATES REMOVED)  
  
=> s l5(s) (synthetas? or synthas?)  
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH  
FIELD CODE - 'AND' OPERATOR ASSUMED 'L42(S) (SYNTHEtas'  
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH  
FIELD CODE - 'AND' OPERATOR ASSUMED 'L44(S) (SYNTHEtas'  
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH  
FIELD CODE - 'AND' OPERATOR ASSUMED 'L48(S) (SYNTHEtas'  
L6      64 L5(S) (SYNTHEtas? OR SYNTHAs?)  
  
=> d ti 16 1-64  
  
L6      ANSWER 1 OF 64 USPATFULL on STN  
TI      Sequence-determined DNA fragments and corresponding polypeptides encoded  
thereby  
  
L6      ANSWER 2 OF 64 USPATFULL on STN  
TI      Nucleic acid and amino acid sequences relating to Enterobacter cloacae  
for diagnostics and therapeutics  
  
L6      ANSWER 3 OF 64 USPATFULL on STN  
TI      Process and materials for production of glucosamine  
  
L6      ANSWER 4 OF 64 USPATFULL on STN  
TI      Listeria monocytogenes genome, polypeptides and uses  
  
L6      ANSWER 5 OF 64 USPATFULL on STN  
TI      Complete genome and protein sequence of the hyperthermophile  
methanopyrus kandleri av19 and monophyly of archael methanogens and  
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L6      ANSWER 6 OF 64 USPATFULL on STN  
TI      Outcome prediction and risk classification in childhood leukemia  
  
L6      ANSWER 7 OF 64 USPATFULL on STN  
TI      System for regulating in vivo the expression of a transgene by  
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L6      ANSWER 8 OF 64 USPATFULL on STN  
TI      Gene expression profiling of colon cancer with DNA arrays  
  
L6      ANSWER 9 OF 64 USPATFULL on STN  
TI      Genetic networks regulated by attenuated GH/IGF1 signaling and caloric  
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L6      ANSWER 10 OF 64 USPATFULL on STN  
TI      Genes of an otitis media isolate of haemophilus influenzae
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- L6 ANSWER 11 OF 64 USPATFULL on STN
TI Signatures of ER status in breast cancer
- L6 ANSWER 12 OF 64 USPATFULL on STN
TI Methods and apparatus for gel-free qualitative and quantitative proteome analysis, and uses therefore
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- L6 ANSWER 14 OF 64 USPATFULL on STN
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TI Methods of screening test compounds using GADD34L, an eIF2alpha-specific phosphatase subunit
- L6 ANSWER 23 OF 64 USPATFULL on STN
TI Nucleic acid sequences relating to Candida albicans for diagnostics and therapeutics
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TI Process and materials for production of glucosamine and N-acetylglucosamine
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TI Methods of diagnosis of ovarian cancer, compositions and methods of screening for modulators of ovarian cancer
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TI Nucleic acid and amino acid sequences relating to Acinetobacter baumannii for diagnostics and therapeutics
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TI Rapid quantitative analysis of proteins or protein function in complex mixtures
L6 ANSWER 57 OF 64 USPATFULL on STN
TI PROCESSES FOR PRODUCING SUGAR NUCLEOTIDES AND COMPLEX CARBOHYDRATES
L6 ANSWER 58 OF 64 USPATFULL on STN
TI Process and materials for production of glucosamine
L6 ANSWER 59 OF 64 USPATFULL on STN
TI Processes for producing sugar nucleotides and complex carbohydrates
L6 ANSWER 60 OF 64 USPATFULL on STN
TI Expressed sequences of arabidopsis thaliana
L6 ANSWER 61 OF 64 CAPLUS COPYRIGHT 2006 ACS on STN
TI Myxospore coat synthesis in Myxococcus xanthus: enzymes associated with uridine 5'-diphosphate-N-acetylgalactosamine formation during myxospore development
L6 ANSWER 62 OF 64 BIOTECHNO COPYRIGHT 2006 Elsevier Science B.V. on STN
TI Subcellular distribution of glycolyltransferases in rodent liver and their significance in special reference to the synthesis of N-glycolylneuraminic acid
L6 ANSWER 63 OF 64 Elsevier BIOBASE COPYRIGHT 2006 Elsevier Science B.V. on STN
TI Mosquito glucosamine-6-phosphate N-acetyltransferase: cDNA, gene structure and enzyme kinetics
L6 ANSWER 64 OF 64 LIFESCI COPYRIGHT 2006 CSA on STN
TI Purification and properties of acetyl-CoA: Glucosamine 6-phosphate N-acetyltransferase from rat liver.

=> d 16 ibib abs 6 17 24 51 54 58

L6 ANSWER 6 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2006:74111 USPATFULL

TITLE: Outcome prediction and risk classification in childhood leukemia

INVENTOR(S): Willman, Cheryl L., Albuquerque, NM, UNITED STATES
Helman, Paul, Albuquerque, NM, UNITED STATES
Veroff, Robert, Albuquerque, NM, UNITED STATES
Mosquera-Caro, Monica, Albuquerque, NM, UNITED STATES
Davidson, George S., Albuquerque, NM, UNITED STATES
Martin, Shawn B., Albuquerque, NM, UNITED STATES
Atlas, Susan R., Albuquerque, NM, UNITED STATES
Andries, Erik, Rio Rancho, NM, UNITED STATES
Kang, Huining, Albuquerque, NM, UNITED STATES
Shuster, Jonathan J., Gainesville, FL, UNITED STATES
Wang, Xuefei, Albuquerque, NM, UNITED STATES
Harvey, Richard C., Placitas, NM, UNITED STATES
Haaland, David M., Albuquerque, NM, UNITED STATES
Potter, Jeffrey W., Albuquerque, NM, UNITED STATES

| | NUMBER | KIND | DATE |
|---------------------|----------------|------|---------------|
| PATENT INFORMATION: | US 2006063156 | A1 | 20060323 |
| APPLICATION INFO.: | US 2003-729895 | A1 | 20031205 (10) |

| | NUMBER | DATE |
|-----------------------|-----------------|---------------|
| PRIORITY INFORMATION: | US 2002-432064P | 20021206 (60) |
| | US 2002-432077P | 20021206 (60) |
| | US 2002-432078P | 20021206 (60) |
| | US 2003-510904P | 20031014 (60) |
| | US 2003-510968P | 20031014 (60) |
| | US 2003-527610P | 20031205 (60) |

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: COLEMAN SUDOL SAPONE, P.C., 714 COLORADO AVENUE, BRIDGE PORT, CT, 06605-1601, US

NUMBER OF CLAIMS: 42

EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 23 Drawing Page(s)

LINE COUNT: 12227

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Genes and gene expression profiles useful for predicting outcome, risk classification, cytogenetics and/or etiology in pediatric acute lymphoblastic leukemia (ALL). OPAL1 is a novel gene associated with outcome and, along with other newly identified genes, represent a novel therapeutic targets.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 17 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2005:49939 USPATFULL
TITLE: Metabolic engineering for enhanced production of chitin and chitosan in microorganisms
INVENTOR(S): Deng, Ming-De, Manitowoc, WI, UNITED STATES
McMullin, Thomas W., Manitowoc, WI, UNITED STATES
Grund, Alan D., Manitowoc, WI, UNITED STATES

| | NUMBER | KIND | DATE |
|---------------------|----------------|------|---------------|
| PATENT INFORMATION: | US 2005042735 | A1 | 20050224 |
| APPLICATION INFO.: | US 2004-823397 | A1 | 20040412 (10) |

| | NUMBER | DATE |
|-----------------------|-----------------|---------------|
| PRIORITY INFORMATION: | US 2003-462087P | 20030411 (60) |
| DOCUMENT TYPE: | Utility | |
| FILE SEGMENT: | APPLICATION | |

LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202

NUMBER OF CLAIMS: 35

EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 1 Drawing Page(s)

LINE COUNT: 6323

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Disclosed is a fermentation method for the production of commercially useful amounts of chitin and/or chitosan by a biosynthetic process. Also disclosed are genetically modified microorganisms useful in such a method and a microbial biomass containing chitin and/or chitosan produced by such a method.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 24 OF 64 USPATFULL on STN

ACCESSION NUMBER: 2004:120566 USPATFULL

TITLE: Process and materials for production of glucosamine and N-acetylglucosamine

INVENTOR(S):
Deng, Ming-De, Manitowoc, WI, UNITED STATES
Angerer, J. David, Hockessin, DE, UNITED STATES
Cyron, Don, Lincoln University, PA, UNITED STATES
Grund, Alan D., Manitowoc, WI, UNITED STATES
Jerrell, Thomas A., JR., Manitowoc, WI, UNITED STATES
Leanna, Candice, Green Bay, WI, UNITED STATES
Mathre, Owen, Wilmington, DE, UNITED STATES
Rosson, Reinhardt, Manitowoc, WI, UNITED STATES
Running, Jeff, Manitowoc, WI, UNITED STATES
Severson, Dave, Two Rivers, WI, UNITED STATES
Song, Linsheng, Manitowoc, WI, UNITED STATES
Wassink, Sarah, Sheboygan, WI, UNITED STATES

| NUMBER | KIND | DATE |
|--------|------|------|
|--------|------|------|

PATENT INFORMATION: US 2004091976 A1 20040513

APPLICATION INFO.: US 2003-612779 A1 20030701 (10)

| NUMBER | DATE |
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PRIORITY INFORMATION: US 2002-393348P 20020701 (60)

DOCUMENT TYPE: Utility

FILE SEGMENT: APPLICATION

LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202

NUMBER OF CLAIMS: 212

EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 25 Drawing Page(s)

LINE COUNT: 15039

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A biosynthetic method for producing glucosamine and N-acetylglucosamine is disclosed. Such a method includes the fermentation of a genetically modified microorganism to produce glucosamine and/or N-acetylglucosamine. Also disclosed are genetically modified microorganisms that are useful for producing glucosamine and N-acetylglucosamine. In addition, methods of recovering N-acetylglucosamine that has been produced by a fermentation process, including methods that result in N-acetylglucosamine of high purity, are described. Also disclosed is a method to produce glucosamine from N-acetylglucosamine.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 51 OF 64 USPATFULL on STN

ACCESSION NUMBER: 2003:64818 USPATFULL

TITLE: Process and materials for production of glucosamine

INVENTOR(S) : Berry, Alan, Manitowoc, WI, UNITED STATES
Burlingame, Richard P., Manitowoc, WI, UNITED STATES
Millis, James R., Kohler, WI, UNITED STATES
PATENT ASSIGNEE(S) : Arkion Life Sciences LLC (U.S. corporation)

| | NUMBER | KIND | DATE |
|-----------------------|---|------|---------------|
| PATENT INFORMATION: | US 2003044939 | A1 | 20030306 |
| APPLICATION INFO.: | US 2001-24460 | A1 | 20011217 (10) |
| RELATED APPLN. INFO.: | Continuation of Ser. No. US 1998-115475, filed on 15 Jul 1998, GRANTED, Pat. No. US 6372457 | | |
| | Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998, UNKNOWN | | |

| | NUMBER | DATE |
|-----------------------|--|---------------|
| PRIORITY INFORMATION: | US 1997-35494P | 19970114 (60) |
| DOCUMENT TYPE: | Utility | |
| FILE SEGMENT: | APPLICATION | |
| LEGAL REPRESENTATIVE: | SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202 | |
| NUMBER OF CLAIMS: | 60 | |
| EXEMPLARY CLAIM: | 1 | |
| NUMBER OF DRAWINGS: | 16 Drawing Page(s) | |
| LINE COUNT: | 4958 | |

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucosamine, as well as recombinant nucleic acid molecules and the proteins produced by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 54 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2002:287596 USPATFULL
TITLE: PROCESS FOR PRODUCTION OF N-GLUCOSAMINE
INVENTOR(S) : BERRY, ALAN, BLOOMFIELD, NJ, UNITED STATES
BURLINGAME, RICHARD P., MANITOWOC, WI, UNITED STATES
MILLIS, JAMES R., KOHLER, WI, UNITED STATES

| | NUMBER | KIND | DATE |
|-----------------------|--|------|--------------|
| PATENT INFORMATION: | US 2002160459 | A1 | 20021031 |
| APPLICATION INFO.: | US 1999-341600 | A1 | 19990915 (9) |
| | WO 1998-US800 | | 19980114 |
| DOCUMENT TYPE: | Utility | | |
| FILE SEGMENT: | APPLICATION | | |
| LEGAL REPRESENTATIVE: | SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202 | | |
| NUMBER OF CLAIMS: | 39 | | |
| EXEMPLARY CLAIM: | 1 | | |
| NUMBER OF DRAWINGS: | 9 Drawing Page(s) | | |
| LINE COUNT: | 2181 | | |

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to a method for producing N-glucosamine by fermentation of a genetically modified microorganism.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 58 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2002:81241 USPATFULL
TITLE: Process and materials for production of glucosamine
INVENTOR(S) : Berry, Alan, Manitowoc, WI, United States

PATENT ASSIGNEE(S) :
Burlingame, Richard P., Manitowoc, WI, United States
Millis, James R., Kohler, WI, United States
Arkion Life Sciences LLC, Wilmington, DE, United States
(U.S. corporation)

| | NUMBER | KIND | DATE |
|-----------------------|--|------|--------------|
| PATENT INFORMATION: | US 6372457 | B1 | 20020416 |
| APPLICATION INFO.: | US 1998-115475 | | 19980715 (9) |
| RELATED APPLN. INFO.: | Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998 | | |

| | NUMBER | DATE |
|-----------------------|--|---------------|
| PRIORITY INFORMATION: | US 1997-35494P | 19970114 (60) |
| DOCUMENT TYPE: | Utility | |
| FILE SEGMENT: | GRANTED | |
| PRIMARY EXAMINER: | Nashed, Nashaat T. | |
| ASSISTANT EXAMINER: | Fronda, Christian L | |
| LEGAL REPRESENTATIVE: | Sheridan Ross P.C. | |
| NUMBER OF CLAIMS: | 59 | |
| EXEMPLARY CLAIM: | 1 | |
| NUMBER OF DRAWINGS: | 21 Drawing Figure(s); 21 Drawing Page(s) | |
| LINE COUNT: | 4835 | |

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucosamine, as well as recombinant nucleic acid molecules and the proteins produced by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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L6 ANSWER 3 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2006:111141 USPATFULL
TITLE: Process and materials for production of glucosamine
INVENTOR(S): Berry, Alan, Manitowoc, WI, UNITED STATES
Burlingame, Richard P., Manitowoc, WI, UNITED STATES
Millis, James R., Kohler, WI, UNITED STATES
PATENT ASSIGNEE(S): Arkion Life Sciences LLC d/b/a Bio-Technical Resources,
Manitowoc, WI, UNITED STATES (U.S. corporation)

| | NUMBER | KIND | DATE |
|-----------------------|---|------|---------------|
| PATENT INFORMATION: | US 2006094085 | A1 | 20060504 |
| APPLICATION INFO.: | US 2005-245473 | A1 | 20051005 (11) |
| RELATED APPLN. INFO.: | Continuation of Ser. No. US 2001-24460, filed on 17 Dec 2001, ABANDONED Continuation of Ser. No. US 1998-115475, filed on 15 Jul 1998, GRANTED, Pat. No. US 6372457 Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998, PENDING | | |

| | NUMBER | DATE |
|-----------------------|--|---------------|
| PRIORITY INFORMATION: | US 1997-35494P | 19970114 (60) |
| DOCUMENT TYPE: | Utility | |
| FILE SEGMENT: | APPLICATION | |
| LEGAL REPRESENTATIVE: | SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202, US | |
| NUMBER OF CLAIMS: | 27 | |
| EXEMPLARY CLAIM: | 1 | |

NUMBER OF DRAWINGS: 16 Drawing Page(s)

LINE COUNT: 4706

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucosamine, as well as recombinant nucleic acid molecules and the proteins produced by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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48 FILE IPA
10 FILE NAPRALERT
506 FILE NLDB

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L3 225 SEA L2 (S) (ACETYLTRANSFERAS?)
L4 134 SEA L3 (S) PHOSPHAT?
L5 95 DUP REM L4 (39 DUPLICATES REMOVED)
L6 64 SEA L5 (S) (SYNTETAS? OR SYNTHAS?)
D TI L6 1-64
D L6 IBIB ABS 6 17 24 51 54 58
D IBIB ABS L6 3

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Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine

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Richard P. Burlingame¹, Alan Berry², Jeffrey A. Running, Candice A. Kunesh,
Linsheng Song, Thomas A. Jerrell, Reinhardt A. Rosson

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Abstract

Glucosamine and *N*-acetylglucosamine are currently produced by extraction and acid hydrolysis of chitin from shellfish waste. Production could be limited by the amount of raw material available and the product potentially carries the risk of shellfish protein contamination. *Escherichia coli* was modified by metabolic engineering to develop a fermentation process. Over-expression of glucosamine synthase (*GlmS*) and inactivation of catabolic genes increased glucosamine production by 15 fold, reaching 60 mg l⁻¹. Since *GlmS* is strongly inhibited by glucosamine-6-P, *GlmS* variants were generated via error-prone PCR and screened. Over-expression of an improved enzyme led to a glucosamine titer of 17 g l⁻¹. Rapid degradation of glucosamine and inhibitory effects of glucosamine and its degradation products on host cells limited further improvement. An alternative fermentation product, *N*-acetylglucosamine, is stable, non-inhibitory to the host and readily hydrolyzed to glucosamine under acidic conditions. Therefore, the glucosamine pathway was extended to *N*-acetylglucosamine by over-expressing a heterologous glucosamine-6-P *N*-acetyltransferase. Using a simple and low-cost fermentation process developed for this strain, over 110 g l⁻¹ of *N*-acetylglucosamine was produced.

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Keywords: Glucosamine; *N*-acetylglucosamine; *Escherichia coli*; *glmS* gene; *GNAI* gene; Metabolic engineering; Fermentation; Lactose induction

1. Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) and its derivative *N*-acetylglucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose) are synthesized in all organisms, including bacteria, yeast, filamentous fungi, plants and animals. In humans, GlcN and GlcNAc are precursors of the disaccharide units in glycosaminoglycans (such as hyaluronic acid, chondroitin sulfate and keratan sul-

fate), which are necessary to repair and maintain healthy cartilage and joint function. Clinical trials with GlcN for treatment of arthritis started in the early 1980s. Since then GlcN has been increasingly used as a dietary supplement (Hungerford and Jones, 2003; Towheed, 2003). It is estimated that 33 million people suffer from osteoarthritis in the United States. The National Institutes of Health (NIH) is conducting a Phase III clinical trial, a multiple-site, double-blind randomized study on the effectiveness of GlcN for treatment of knee osteoarthritis (NIH GlcN/Chondroitin Arthritis Intervention Trial (GAIT); <http://clinicaltrials.gov/show/NCT00032890>). Results of the study are expected to be published in November 2005.

Currently, GlcN is produced by acid hydrolysis of chitin (a linear polymer of GlcNAc) extracted from crab

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and shrimp shells. Concentrated hydrochloric acid breaks down the polymer and deacetylates GlcNAc to form GlcN (Novikov and Ivanov, 1997). GlcNAc is produced by chemical acetylation of GlcN using acetic anhydride (He et al., 2001). GlcN production could become limited by variable raw material supply as the demand continues to increase. Moreover, GlcN from shellfish may not be suitable for people with shellfish allergies. Some filamentous fungi contain chitosan (a linear co-polymer of GlcN and GlcNAc, with GlcN accounting for over 65%) in their cell walls (Velichkov and Georgiev, 1991). The amount of chitosan in fungal biomass is low, generally less than 25% of dry cell weight. Current economics limit GlcN production to the use of fungal biomass from fermentation processes dedicated to citric acid or other primary products.

In the present report, a new microbial fermentation for production of high-quality and low-cost GlcN and GlcNAc was developed. The strategy to develop an economically viable fermentation process was to genetically engineer a high-performance production strain. Pathways for the synthesis and metabolism of GlcN are well characterized in the bacterium *Escherichia coli* and other organisms (Plumbridge, 1995; Plumbridge and Pellegrini, 2004). As shown in Fig. 1, GlcN synthase (encoded by *glmS*) converts fructose-6-phosphate (fruc-

tose-6-P) to GlcN-6-P using glutamine as an amino donor. For the synthesis of peptidoglycan and lipopolysaccharide, essential components of the cell wall of gram-negative bacteria, GlcN-6-P is converted to GlcN-1-P by phosphoglucosamine mutase (encoded by *glmM*). The product is further converted by a bifunctional enzyme, GlcN-1-P N-acetyltransferase/GlcNAc-1-P uridylyltransferase (encoded by *glmU*), to GlcNAc-1-P and then to UDP-GlcNAc.

GlcN and GlcNAc also serve as alternative carbon and nitrogen sources. GlcN is transported and phosphorylated by a mannose transporter (II^{Man} , encoded by the operon *manXYZ*) and the glucose transporter (II^{Glc} , encoded by *ptsG*). GlcNAc is transported and phosphorylated by the mannose transporter and a GlcNAc-specific transporter (II^{NAG} , encoded by *nagE*). GlcNAc-6-P deacetylase (encoded by *nagA*) converts GlcNAc-6-P to GlcN-6-P, which is further converted to fructose-6-P by GlcN-6-P deaminase (encoded by *nagB*). In the *E. coli* genome, *glmS* and *glmU* constitute the operon *glmUS* while *nagA*, -*B*, -*C*, -*D* and -*E* form a regulon (Plumbridge, 1995). The *nagC* gene encodes a regulatory protein that functions as a repressor of the *nag* regulon as well as both an activator and repressor of the *glmUS* operon. The function of *nagD* is not known but is likely related to amino sugar metabolism, as it resides in the *nag* regulon.

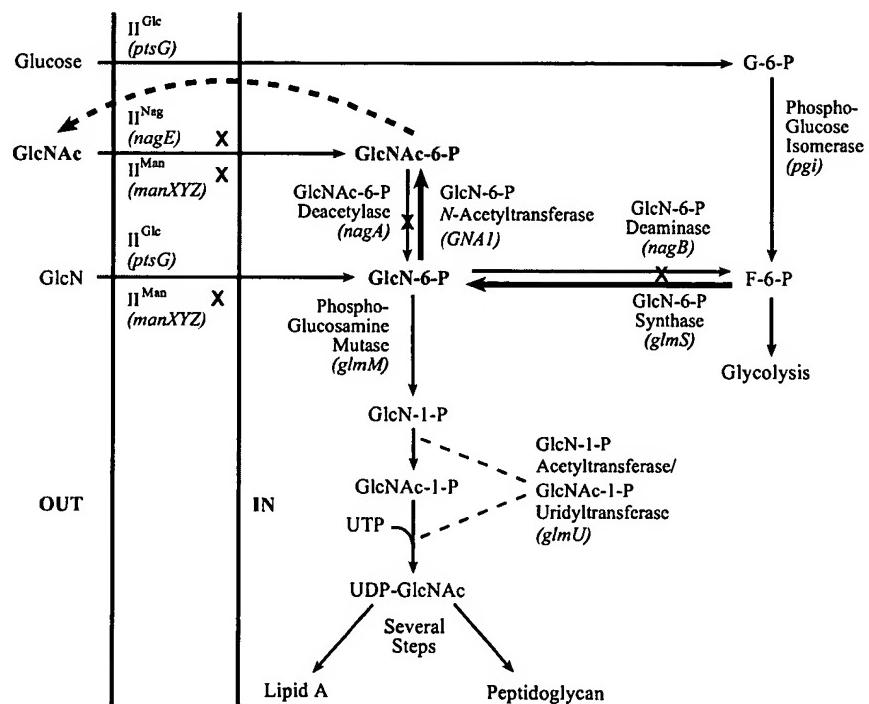


Fig. 1. Pathway engineering for GlcNAc production in recombinant *E. coli*. Crosses indicate metabolic flux blocked by gene inactivation or deletion. Thicker lines indicate introduction and/or increase of the metabolic flux by gene over-expression. Dephosphorylation and secretion of GlcNAc-6-P are indicated as a dotted line. G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, GlcN: glucosamine, GlcNAc: *N*-acetylglucosamine, II^{Glc} : glucose transporter, II^{NAG} : GlcNAc-specific transporter, II^{Man} : mannose transporter.

Both the synthetic and degradative pathways for GlcN and GlcNAc are tightly regulated. In the present work, metabolic engineering was used to develop *E. coli* strains for production by fermentation of the monomers of GlcN and GlcNAc. Strain and process optimization led to the development of an economically competitive process.

2. Materials and methods

2.1. Development of the *E. coli* strain 7101-17(DE3)

E. coli K-12 (ATCC 25947, a derivative of W3110) (Bachmann, 1996) was used as the metabolic engineering host to produce GlcN and GlcNAc. The strain was modified by P1 phage transduction (Miller, 1992) to introduce a mutation or deletion in operons involved in the transport and catabolism of GlcN and GlcNAc. A mutation in *manXYZ* was introduced using phage grown on *E. coli* strain IBPC566 (courtesy of Dr. Plumbridge, Institut de Biologie Physico-Chimique, Paris, France) which contained a tetracycline resistance determinant (*ter*^R from transposon Tn10) linked to the *manXYZ* mutation (designated *manXYZ8 zdj-225::Tn10*) (Plumbridge, 1990). The resulting strain 7101-6 grew poorly on mannose and failed to grow with GlcN as the sole carbon source. In *E. coli* strain IBPC590 (courtesy of Dr. Plumbridge) the entire *nagBACD* operon and a portion of *nagE* was replaced by a *tet*^R determinant (Plumbridge, 1992). In order to use tetracycline selection to introduce the *nag* deletion, strain 7101-6 was plated onto TCS medium which selects for tetracycline-sensitive mutants as described by Maloy and Nunn (1981). Southern blot hybridization showed that the resistance was lost by the deletion of the *tet*^R marker in strain 7101-13. The *Δnag::tet*^R mutation in IBPC590 was introduced by P1 phage transduction into 7101-13 to generate strain 7101-17 (*manXYZ8, Δnag::tet*^R). The defective λDE3 prophage containing a T7 RNA polymerase gene under *lacUV5* promoter control was introduced into 7101-17 using the λDE3 Lysogenization Kit (Novagen, Inc. Madison, WI), generating a T7 expression host, 7101-17(DE3).

2.2. Cloning and integration of the *E. coli* *glmS* gene

The *E. coli* *glmS* gene (Walker et al., 1984) was amplified from *E. coli* genomic DNA by Polymerase Chain Reaction (PCR). The forward primer Up1 (5'-CGG TCT CCC ATG TGT GGA ATT GTT GGC GC-3') contained sequences corresponding to the first 22 nucleotides of the *glmS* coding sequence and a *Bsa*I restriction site. The reverse primer Lo8 (5'-CTC TAG AGC GTT GAT ATT CAG TCA ATT ACA AAC A-3') contained sequences corresponding to positions

between 145 and 177 bases downstream of the *glmS* stop codon and an *Xba*I site. PCR amplification generated a *Bsa*I–*Xba*I fragment containing the *glmS* gene with 177 bp of downstream sequence, including the *glmS* terminator, between positions 6 and 43 following the stop codon (Gay et al., 1986). The PCR product was cloned as a blunt-end fragment into pPCR-Script™Amp SK(+) (Stratagene Cloning System, La Jolla, CA). In the recombinant plasmid a unique *Sac*I restriction site was located 215 bp downstream of the *glmS*.stop codon. The *glmS* sequence was isolated as a *Bsa*I–*Sac*I fragment and cloned between *Nco*I and *Sac*I sites of the expression vector pET24d(+) (Novagen) to generate plasmid pKLN23-23. Cloning in this manner resulted in a *T7lac–glmS* expression cassette containing the *glmS* terminator.

The *T7lac–glmS* cassette was integrated into the *E. coli* chromosome in 7101-17(DE3) by a multistep process. First, the cassette was excised from pKLN23-23 as a *Bgl*II–*Hind*III fragment by a partial digestion with *Bgl*II and a complete digestion with *Hind*III. The plasmid contained three *Bgl*II sites, one located approximately 20 bp upstream of the T7 promoter, and two in *glmS*. The unique *Hind*III site is located 224 bp downstream of the *glmS* stop codon. The expression cassette was cloned between the unique *Bam*HI and *Hind*III sites of plasmid pBRINT-Cm, an integrative vector containing a chloramphenicol selection marker (Cm) at the *lacZ* deletion site (Balbas et al., 1996). This resulted in plasmid pKLN23-28 containing the *T7lac–glmS–Cm* cassette flanked by the 5'- and 3'-termini of the *E. coli lacZ* sequence. The cassette was oriented in the same direction as the *lac* operon.

E. coli strain ATCC 47002 contains mutations that result in the inability to maintain plasmid pBRINT-Cm, which contains a ColE1 origin of replication. Cells were transformed with pKLN23-28 and selection with chloramphenicol resulted in strains that contained a *T7lac–glmS–Cm* cassette integrated at the *lacZ* deletion site in the chromosome. The cassette was then transferred by P1 phage transduction into strain 7101-17 (DE3), generating strain 2123-12 (*manXYZ8, Δnag::tet*^R DE3, *ΔlacZ::T7lac–glmS–Cm*).

2.3. Generation of *GlmS* mutants resistant to product inhibition

Variants of *glmS* genes were generated by error-prone PCR using pKLN23-28 as template DNA. PCR conditions were essentially as described by Moore and Arnold (1996). PCR products were cloned into the backbone of pKLN23-28, resulting in a library of *T7lac* expression vectors containing various *glmS* mutants. Following transformation into the expression host 7101-17(DE3), clones with increased GlcN production were screened by a plate assay. This was done by stabbing

individual colonies into M9A plates which had been overlayed with soft agar containing cells of an *E. coli* GlcN auxotrophic strain as indicator. After overnight incubation, the halo size (growth area of indicator cells) reflected the level of GlcN production. Improved *glmS* genes were integrated into the *E. coli* chromosome as described above. Strain 2123-54 contains a *T7lac* expression cassette with a mutant *glmS* gene (*glmS*54*). PCR mutagenesis, mutant screening and characterization will be detailed elsewhere.

2.4. Integration of the *T7lac–glmS*54* expression cassette at the *galK* site

By using a temperature shift protocol described by Hamilton et al. (1989), the *T7lac–glmS*54* expression cassette was integrated at the *galK* site in 7101-17(DE3), generating strain 7107-18. For that purpose, vector pSW07-9 was constructed with a kanamycin resistance marker (*kan^R*), a temperature-sensitive pSC101 replication origin and the *T7lac–glmS*54* expression cassette flanked by the *E. coli gal* sequence. A portion of the *gal* operon (*galTKM*, 3.3 kb, starting at 14 bp upstream of the ATG start codon of *galT* and ending at 68 bp downstream of the stop codon of *galM*) was amplified by PCR from *E. coli* W3110 genomic DNA and cloned into vector pPCR-ScriptTM Amp SK(+). A 0.7-kb deletion was made in the *galK* sequence (between the restriction sites *Sfi*I and *Mlu*I) and restriction sites (*Mlu*I, *Sal*I, *Bgl*II, and *Msc*I) were added at the deletion site, generating plasmid pKLN07-1. A kanamycin resistance marker was isolated as a *Pst*I fragment from pUC4K (Amersham Pharmacia Biotech, Piscataway, NJ) and ligated into the *Not*I site of the plasmid pKLN07-1 after both were blunted with T7 DNA polymerase. The orientation of the *kan^R* marker in the resulting plasmid was not determined. The *kan^R–galTKM* sequence was isolated as a *Bam*HI/*Sac*II fragment, blunted with T4 DNA polymerase and ligated with a *Pvu*II/*Sma*I fragment containing the temperature-sensitive replication origin of pMAK705 (Hamilton et al., 1989), generating plasmid pSW07-4. Lastly, the expression cassette *T7lac–glmS*54*, including the *glmS* terminator, was isolated from pKLN23-54 as a *Not*I fragment, blunted with T4 DNA polymerase, and cloned into pSW07-4 at the *Msc*I site, generating plasmid pSW07-9. The *T7lac–glmS*54* cassette was placed in the same orientation as the *gal* operon. The plasmid was transformed into 7101-17(DE3). Sequence integration and subsequent removal of the vector sequence were carried out as described previously (Hamilton et al., 1989). The resulting strain 7107-18 was confirmed by Southern blot to contain the *T7lac–glmS*54* expression cassette integrated at the *galK* deletion site. The expression cassette was oriented in the same direction as the *gal* operon.

2.5. Cloning and expression of different *GNA1* genes

Forward and reverse primers were designed to amplify by PCR the GlcN-6-P N-acetyltransferase genes from *Saccharomyces cerevisiae* (*ScGNA1*), *Candida albicans* (*CaGNA1*) and *Arabidopsis thaliana* (*AtGNA1*) according to the sequence information available at the web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The DNA templates used in PCR were from *S. cerevisiae* strain S288C, *C. albicans* strain ATCC10261 and *A. thaliana* BAC clone F14F8 (*Arabidopsis* Biological Resource Center DNA Stock Center, Columbus, OH). All the forward primers contained a *Bsa*I site and 21–31 nucleotides coding for the N-terminal peptide sequences, including the ATG start codon. All the reverse primers contained an *Xho*I site and 24–30 nucleotides coding for the C-terminal peptide sequences, starting from their stop codon.

ScGNA1 forward primer: 5'-GAT CGG TCT CGC ATG AGC TTA CCC GAT GGA TTT TAT ATA AGG C-3'.

ScGNA1 reverse primer: 5'-GAT CCT CGA GCT ATT TTC TAA TTT GCA TTT CCA CGC CTG C-3'.

CaGNA1 forward primer: (5'-GAT CGG TCT CGC ATG ATG TTA CCA CAA GGT TAT AC-3'.

CaGNA1 reverse primer: 5'-GAT CCT CGA GCT AGA ATC TAC ATA CCA TTT CAA C-3'.

AtGNA1 forward primer: 5'-GAT GGT CTC GCA TGG CTG AGA CAT TCA AGA TC-3'.

AtGNA1 reverse primer: 5'-GAT CCT CGA GTT AAT CGA AGT ACT TAG ACA TTT GAA TC-3'.

PCR amplifications were conducted using the standard protocol to generate DNA fragments containing different *GNA1* coding sequences flanked by *Bsa*I and *Xho*I sites. The sequences were cloned between the *Nco*I and *Xho*I sites in the expression vector pET24d(+), creating plasmids with expression cassettes *T7lac–ScGNA1* (pSW07-62), *T7lac–CaGNA1* or *T7lac–AtGNA1*.

2.6. Integration of multiple copies of *T7lac–ScGNA1* expression cassette in the chromosome

Using cloning strategies and temperature selection protocols described above, the *T7lac–ScGNA1* expression cassette was integrated at the *manXYZ* locus, generating strain 7107-92. The expression cassette was isolated as an *Nae*I fragment from plasmid pSW07-62, starting at position 49 upstream of the T7 promoter and ending at position 164 downstream of the T7 terminator. At the insertion site, most of the *manXYZ* sequence was deleted by the removal of a 2647-bp *Hpa*I fragment.

To maximize *GNA1* expression, three additional copies of *T7lac–ScGNA1* cassette were integrated at the loci of *fucIK*, *treB* and *melRAB*, which encode for enzymes involved in the metabolism of L-fucose,

trehalose and melibiose, respectively. The target sites were chosen because these carbohydrates serve as alternative carbon sources. Although trehalose was also known to act as an osmoprotectant in *E. coli*, deletion and insertion at the *treB* locus did not noticeably affect growth and GlcNAc production in media containing glucose as the sole carbon source, as compared to strains with the wild type *tre* operon. The second copy of the cassette was integrated at the *fucIK* site, replacing a portion of the *fucI* and *fucK* genes (a 1239-bp *HpaI*–*BsrGI* fragment), generating strain 7107-607. A *BglII*–*BsrGI* fragment of 149 bp in the *treB* coding sequence was deleted and replaced by the third copy of the *GNA1* cassette, generating strain 7107-608. A different strain with three copies of *GNA1* (strain 7107-609) was constructed by integrating the third copy of the cassette at the *melRAB* deletion site. The entire *melA* and the first 199 nucleotides of the *melB* sequences were deleted by the removal of a 1676-bp *BglII*–*AsiSI* fragment. Using 7107-608 as the parent strain, a fourth copy of the cassette was integrated at the *melRAB* site. All integrated copies of the *T7lac*–*ScGNA1* cassette were oriented in the same direction as the targeted operons.

2.7. Growth medium

The basic growth media used for GlcN or GlcNAc production were defined mineral salt media, M9A or M9B. M9A medium included the following macro-elements (per liter): 14 g KH₂PO₄, 16 g K₂HPO₄, 1 g Na₃Citrate·2H₂O, 7.5 g (NH₄)₂SO₄, 0.25 g MgSO₄·7H₂O and 0.015 g CaCl₂·2H₂O. The pH was between 6.8 and 7.0 after sterilization. M9B medium contained (per liter) 6 g KH₂PO₄, 24 g K₂HPO₄, 1 g Na₃Citrate·2H₂O, 10 g (NH₄)₂SO₄, 0.6 g MgSO₄·7H₂O and 0.025 g CaCl₂·2H₂O. The pH was between 7.0 and 7.2 after sterilization. Unless specified otherwise, the trace elements were supplied as follows (per liter, final concentration): 0.01 mg CoCl₂·6H₂O, 0.01 mg H₃BO₃, 0.01 mg CoSO₄·5H₂O, 0.5 mg FeSO₄·7H₂O, 0.033 mg MnSO₄·H₂O, 0.38 mg ZnSO₄·7H₂O and 0.01 mg Na-MoO₄·2H₂O. The media were supplemented with glucose (20 g l⁻¹) as the carbon source, if not indicated otherwise.

2.8. Shake flask culture

A three-step protocol was developed to evaluate different production strains in shake flask experiments. First, cells freshly grown on LB plates were used to start cultures in 3 ml LB, which were grown at 37 °C for about 8 h. Secondly, a portion of the cultures (1.5 ml) was used to inoculate 50 ml of M9A medium in 250-ml shake flasks. Cultures were incubated at 37 °C and shaken at 225 rpm for about 16 h. Cell densities of the cultures

were measured at 600 nm (OD₆₀₀). One unit of OD₆₀₀ corresponded approximately to a cell density of 0.5 g l⁻¹ dry weight. This culture step was included for cells to adapt to the minimal medium and for reproducible results of GlcN or GlcNAc production. Next, aliquots of the cultures were added to 50 ml of M9A or M9B containing IPTG (0.2 mM) in 250-ml shake flasks. The initial values of OD₆₀₀ were set at 0.3. Cells were incubated at 37 °C and shaken at 225 rpm for 72 h. Samples of 1 ml were taken at 24, 48 and 72 h to measure cell density and levels of glucose, acetate, GlcN and/or GlcNAc. At 24 and 48 h, pH was adjusted to 7.0 by addition of a small amount of 10 M NaOH and the glucose level in the medium was adjusted to 20 g l⁻¹ by addition of a concentrated glucose solution (65%, w/v).

2.9. GlcN production in 1-L fermentors

Fed-batch fermentation was run in 1-L fermentors. Fermentation medium contained the following components (per liter): 3.5 g KH₂PO₄, 4 g K₂HPO₄, 1 g Na₃Citrate·2H₂O, 5 g (NH₄)₂SO₄, 0.6 g MgSO₄·7H₂O, 0.05 g CaCl₂·2H₂O and 0.25 g antifoam agent Mazu DF204. These components were batch sterilized together. The pH was between 6.8 and 7.0 after sterilization. Unless specified otherwise, the same trace elements described above were added, but at 10-fold higher concentrations. A glucose solution (65%, w/v) was sterilized separately and added into the medium to the concentrations of 2–5 g l⁻¹ before inoculation.

The medium volume before inoculation was 50% of the 1-L total fermentor capacity. Aeration was kept between 0.5 to 1 vvm. Dissolved oxygen was maintained at 20% saturation by controlling the rate of agitation. The growth temperature was maintained at 30 or 37 °C, and post-induction temperature at 25 or 30 °C (depending on the experiment). pH was controlled at 6.9 by automatic addition of 10 M NH₄OH, which also provided additional nitrogen for biomass and GlcN production.

Fermentors were inoculated with 2.5–5% culture (by volume) to an initial OD₆₀₀ of about 0.5. Glucose was fed as a 65% solution (w/v) using a computerized program to control growth and minimize production of acetate. When an OD₆₀₀ of 20–30 was reached, the culture was induced with lactose, either by a single point addition or slow feeding (up to 25 g l⁻¹ lactose). Glucose was fed thereafter at a rate of 4–6.5 g l⁻¹ h⁻¹ to supply carbon for GlcN production. With glucose feed and base addition the final volume of the fermentation reached 75–80% of the total fermentor capacity. The fermentation was discontinued 72 h after inoculation.

2.10. GlcNAc production in 1-L fermentors

High GlcNAc titers were achieved by optimizing medium composition, lactose induction (timing and

dose) and glucose feeding. With respect to the protocol described above, a few changes were made. The optimized medium composition was as follows (per liter): 6.67 g KH_2PO_4 , 3.55 g citric acid · H_2O , 0.025 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 2.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.25 g antifoam agent Mazu DF204. These components were batch sterilized together before adding the following trace elements (per liter): 0.1 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.33 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 3.8 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Glucose (65%, w/v) was sterilized separately and added to the medium (5 g per liter) before inoculation. The pH was near 3.0 after sterilization and was adjusted to 6.9 with 10 M NH_4OH before inoculation.

Fermentors were inoculated with 2.5–5% culture (by volume) to an initial OD_{600} of about 0.5% and 65% w/v glucose was fed on a program to limit growth to a rate of 0.4–0.45 h^{-1} . Throughout the fermentation, temperature was maintained at 37 °C. The pH was controlled at 6.9 by addition of 10 M NH_4OH . When an OD_{600} of 10 was reached, glucose feeding was set at a steady rate of 6.5 $\text{g l}^{-1} \text{h}^{-1}$ (based on initial volume) until the end of fermentation (for a total of 72 h). When an OD_{600} of 25–30 was reached, the culture was induced with a single addition of 5 g l^{-1} lactose without suspension of the glucose feed.

2.11. Enzyme activity assays

GlcN-6-P synthase activity in crude enzyme extracts was measured by a coupled spectrophotometric assay using glutamate dehydrogenase as described previously (Badet et al., 1987). GlcN-6-P N-acetyltransferase was assayed by quantifying the amount of CoA released from acetyl-CoA following the method described by Mio et al. (1999).

2.12. GlcN and GlcNAc determination

GlcN in the culture medium was assayed by a modification of the colorimetric method of Elson and Morgan using 4-dimethylaminobenzaldehyde (Ehrlich's Reagent), essentially as described by Zalkin (1985). GlcNAc interfered with the colorimetric assay for GlcN. In samples which contained a significant amount of GlcNAc, GlcN was assayed by HPLC (Wayne et al., 2000) on a Phenomenex Prodigy ODS(3) C18 column (150 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA) maintained at ambient temperature. The mobile phase was a MeOH:aqueous buffer (1:4 v/v) run at 0.7 ml min^{-1} . The aqueous buffer consisted of 10 mM sodium acetate and 10 mM sodium octanesulfonate, pH 5.1. GlcNAc was measured by HPLC using a Bio-Rad AMINEX HPX 87H Organic Analysis Column (300 × 7.8 mm) that was water-jacketed to maintain the

column temperature at 40 °C. The mobile phase of 0.013 M nitric acid was run at 0.8 ml min^{-1} .

2.13. Determination of GlcN stability

GlcN stability was measured in uninoculated M9A medium supplemented with 40 g l^{-1} glucose. GlcN was prepared as a 30% GlcN hydrochloride solution and was added to the desired final concentrations (50 ml total volume in 250-ml flasks). The pH of the medium was adjusted to pH 7.0, 6.5, 6.0 and 5.5. Flasks were incubated at 30 °C on a shaker and agitated at about 225 rpm. Samples of 1 ml were taken at intervals for analysis. After each sampling, pH in the flasks was measured by submerging a pH probe in the liquid and adjusted to the initial values by adding droplets of a 5-N NaOH solution. GlcN levels were determined by the colorimetric method.

2.14. Effects of GlcN on *E. coli* strain 7107-18

Cells were inoculated into M9A medium supplemented with glucose (40 g l^{-1}) and GlcN at different concentrations: 0, 10, 20 and 40 g l^{-1} . Cell density (OD_{600}) and numbers of colony-forming units were determined in samples taken at intervals. To test the involvement of GlcN degradation products in growth inhibition, growth medium containing different levels of GlcN were prepared and incubated at 30 °C for 4, 8 and 16 h to allow degradation products to form. Cells were inoculated into the media and cell growth in these cultures was compared to cultures that were inoculated immediately (within 30 min) after the media had been prepared.

2.15. GlcNAc hydrolysis

The conversion of GlcNAc to GlcN was examined using a 1% solution of GlcNAc incubated at various temperatures (35, 60 and 100 °C) in the presence of HCl (0–1.0 M). Samples were taken over time and analyzed by HPLC for GlcNAc and GlcN.

3. Results

3.1. Gene inactivation and *glmS* over-expression

An *E. coli* K-12 strain (ATCC 25947) was used as the host for metabolic engineering. The genotypes of the host strain and key recombinant strains are listed in Table 1. The host strain was modified to minimize the transport and catabolism of GlcN and GlcNAc. This was achieved by introducing a mutation in the *manXYZ* operon and deleting the *nag* regulon (Fig. 1). A λ-DE3 element containing a T7 RNA polymerase gene under

Table 1
E. coli strains

| Strain no. | Genotype | Remark | Reference |
|--------------------------|--|--|-------------------|
| ATCC 25947 ¹⁾ | F ⁻ λ ⁻ rph-1 IN(rrnD-rrnE)1 Thy, a derivative of <i>E. coli</i> K-12 strain W3110 | Parent strain for pathway engineering | ATCC |
| IBPC566 | thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 manXYZ8 zdj-225::Tn10 | | Plumbridge (1990) |
| IBPC590 | thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 Δnag::tet ^R | | Plumbridge (1992) |
| ATCC 47002 | F ⁻ recB21 recC22sbcB15 leu-6 ara-14 his-4 λ ⁻ | | ATCC |
| 7101-17 (DE3) | Δnag::tet ^R manXYZ8 DE3 | Derived from ATCC 25947 | This work |
| 2123-12 | Δnag::tet ^R manXYZ8 DE3 ΔlacZ::T7lac-glmS-Cam ^R | Over-expression of the <i>E. coli</i> glmS gene integrated at the lacZ locus, derived from 7101-17(DE3) | This work |
| 2123-54 | Δnag::tet ^R manXYZ8 DE3 ΔlacZ::T7lac-glmS*54-Cam ^R | Over-expression of a mutant <i>E. coli</i> glmS gene integrated at the lacZ locus, derived from 7101-17(DE3) | This work |
| 7107-18 ²⁾ | Δnag::tet ^R manXYZ8 DE3
ΔgalK::T7lac-glmS*54 | Over-expression of a mutant <i>E. coli</i> glmS gene integrated at the galK locus, derived from 7101-17(DE3) | This work |
| 7107-88 | 7107-18, pET24d(+) Kan ^R | Hosting an empty vector | This work |
| 7107-87 | 7107-18, pET24d(+)T7lac-ScGNA1 Kan ^R | Hosting an expression vector containing the <i>Saccharomyces cerevisiae</i> GNA1 gene | This work |
| 7107-93 | 7107-18, pET24d(+)T7lac-AtGNA1 Kan ^R | Hosting an expression vector containing the <i>Arabidopsis thaliana</i> GNA1 gene | This work |
| 7107-117 | 7107-18, pET24d(+)T7lac-CaGNA1 Kan ^R | Hosting an expression vector containing the <i>Candida albicans</i> GNA1 gene | This work |
| 7107-92 | 7107-18, ΔmanXYZ::T7lac-ScGNA1 | One copy of integrated ScGNA1 expression cassette | This work |
| 7107-607 | 7107-18, ΔmanXYZ::T7lac-ScGNA1
ΔfucIK::T7lac-ScGNA1 | Two copies of integrated ScGNA1 expression cassette | This work |
| 7107-608 | 7107-18, ΔmanXYZ::T7lac-ScGNA1
ΔfucIK::T7lac-ScGNA1
ΔtreB::T7lac-ScGNA1 | Three copies of integrated ScGNA1 expression cassette | This work |
| 7107-609 | 7107-18, ΔmanXYZ::T7lac-ScGNA1
ΔfucIK::T7lac-ScGNA1
ΔmelAB::T7lac-ScGNA1 | Three copies of integrated ScGNA1 expression cassette | This work |
| 7107-612 | 7107-18, ΔmanXYZ::T7lac-ScGNA1
ΔfucIK::T7lac-ScGNA1
ΔtreB::T7lac-ScGNA1
ΔmelAB::T7lac-ScGNA1 | Four copies of integrated ScGNA1 expression cassette | This work |

Note: (1) Except IBPC566, IBPC590 and ATCC47002, all other strains listed in the table were derived from the same parent strain ATCC 25947.

(2) Strains listed below this lane were all derived from strain 7107-18. For clarity, the genotypes of these strains are shown as 7107-18 plus additional modifications.

control of the *lacUV5* promoter was integrated in the chromosome by lysogenization so that the T7-based expression system could be used to over-express biosynthesis genes (Studier and Moffatt, 1986), generating strain 7101-17(DE3). The *E. coli* glmS coding sequence was cloned and placed under *T7lac* promoter control, forming a *T7lac-glmS* expression cassette in plasmid pET24d(+). The expression cassette was then integrated into the chromosome of the strain 7101-17(DE3) at the *lacZ* locus, generating strain 2123-12. Different *E. coli* strains were grown at 37 °C in the defined mineral salt medium M9A containing 1 mM isopropylthio-β-D-galactoside (IPTG). In strain 2123-12 GlmS protein was expressed as a predominant protein as observed on SDS-PAGE (data not shown). GlmS

activity in the parent strain 7101-17(DE3) was very low (around 10 nmol min⁻¹ mg⁻¹ protein) while it reached about 500 nmol min⁻¹ mg⁻¹ protein in 2123-12. The amount of GlcN detected in the growth medium was typically 4 mg l⁻¹ with the wild type *E. coli* strain at 26 h. The level reached 75 mg l⁻¹ with strain 2123-12.

3.2. Generation and over-expression of GlcN synthases resistant to product inhibition

Mutant variants of the *E. coli* glmS gene were generated by error-prone PCR. Improved enzymes were identified by screening for higher GlcN producers using a cross-feeding plate assay. From a population of about 9000 clones, 84 clones were confirmed to produce GlcN

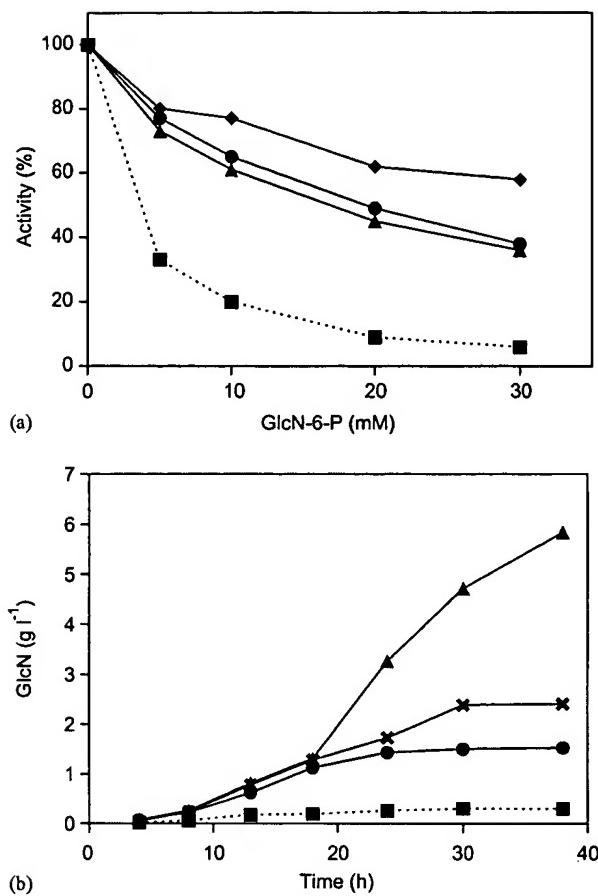


Fig. 2. *GlmS* mutants resistant to product inhibition. Strain 2123-12 (—■—, dotted line) is a strain containing an integrated *T7lac-glmS* expression cassette (with the wild type *E. coli* *glmS* gene). Other *E. coli* strains contain an integrated expression cassette with an *E. coli* *glmS* mutant gene generated through error-prone PCR: 2123-49 (—●—), 2123-51 (—×—), 2123-54 (—▲—) and 2123-124 (—◆—). (a) *GlmS* activity determined in the presence of various concentrations of GlcN-6-P. (b) GlcN production in shake flask cultures induced with 1 mM IPTG at time of inoculation.

at levels significantly higher than strain 2123-12. Expression constructs with mutant *glmS* genes (*glmS**) were integrated into the chromosome at the *lacZ* site. Several mutant *GlmS* enzymes showed significantly reduced sensitivity to product inhibition (Fig. 2a). One of the best mutant strains, 2123-54, over-expressing the mutant *glmS* gene (*glmS*54*), produced 6 g l⁻¹ GlcN (Fig. 2b). This was 20-fold higher than strain 2123-12 that over-expressed the wild-type *E. coli* *glmS* gene.

3.3. Lactose induction of GlcN production

In strain 2123-54 the *T7lac-glmS*54* expression cassette was integrated into the chromosome at the *lacZ* locus, resulting in inactivation of the *lac* operon. Genes under T7 promoter control require induction of the T7 RNA polymerase that is under *lacUV5* promoter

control in the DE3 element. GlcN production in this strain was inducible by IPTG, which is too expensive to be used in large-scale fermentation. A more cost-effective alternative is to use lactose as an inducer. This requires a functional LacZ enzyme (β -galactosidase) to convert lactose to allolactose, the true inducer. Therefore, strain 7107-18 was developed by integrating the *T7lac-glmS*54* cassette at an alternate integration site. The *galK* locus was chosen as the integration site so that galactose could also be used as inducer (Mattanovich et al., 1998).

Glucose is often the preferred low-cost carbon source in industrial scale fermentations. Lactose induction with the T7 expression system is subject to repression by glucose. This problem can usually be minimized by appropriate process conditions (Neubauer et al., 1992; Hoffman et al., 1995). Lactose induction of GlcN production was studied in 1-L fermentors. Induction after an initial growth phase was found to be most effective for GlcN production. Cells were grown with glucose to an OD₆₀₀ of about 20. The feed was switched from glucose to lactose for about 10 h, after which glucose feeding was resumed. Using this protocol, GlcN levels reached 17 g l⁻¹ by 72 h (Fig. 3a).

3.4. Degradation of GlcN and effects of GlcN on cell growth

Further GlcN titer improvement was impaired by a number of factors. These included GlcN instability as well as inhibition of cell growth by GlcN and its degradation products. GlcN degradation was found to be strongly pH-dependent, occurring faster at higher pH (Fig. 4a). When GlcN (60 g l⁻¹) was incubated at 30 °C in an uninoculated M9A medium adjusted to pH 7.0, as much as 68% of GlcN was degraded within 52 h. GlcN was stable when pH was below 4.8. An amber color developed as GlcN degraded.

Inhibitory effects of GlcN and its degradation product(s) on *E. coli* 7107-18 cells were demonstrated by growing cells in a medium containing different levels of GlcN (Fig. 4b). Growth was slightly inhibited by GlcN at 10 and 20 g l⁻¹. GlcN at 40 g l⁻¹ completely prevented cell growth. After 16 h of incubation with GlcN, numbers of colony-forming units declined, indicating cell death. After 52 h, the viable cell count leveled off at about one-fifth of the original number (data not shown). When cells were inoculated in media which had been pre-incubated with GlcN at 35 g l⁻¹ for 8 h, growth inhibition and cell death were more severe, suggesting that GlcN degradation product(s) are even more inhibitory to the host cell than GlcN.

To minimize product loss due to degradation, GlcN production at low pH was evaluated. *E. coli* cells were grown at normal optimal pH (6.5–7.0), induced with lactose and then switched to pHs below 5.0. This

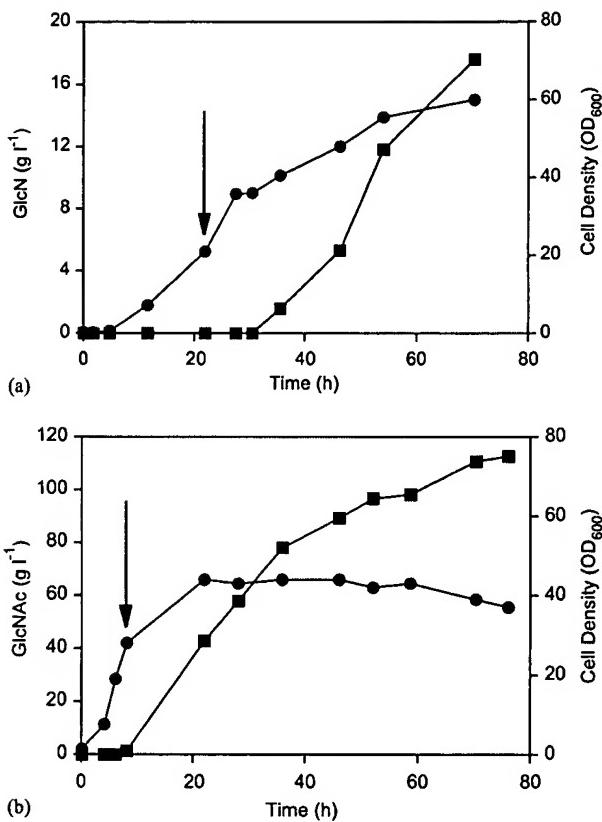


Fig. 3. Production of GlcN and GlcNAc in one-liter fermentors. Fermentations were run in a fed-batch mode. Cell density (—●—, OD₆₀₀), GlcN and GlcNAc titers (—■—) were monitored. (a) GlcN production using *E. coli* strain 7107-18 containing an integrated *T7lac-glmS*54* expression cassette. Cells were grown to an OD₆₀₀ of 22 and induced by lactose (30 g l⁻¹) added during a 10-h period as shown by the arrow. (b) GlcNAc production using *E. coli* strain 7107-607 containing an integrated *T7lac-glmS*54* expression cassette and two integrated copies of a *T7lac-ScGNA1* expression cassette. Cells were grown to an OD₆₀₀ of 27 and induced with lactose (5 g l⁻¹) added as a single pulse as shown by the arrow.

improved GlcN production by about 20% (data not shown).

3.5. Over-expression of GlcN-6-P N-acetyltransferase and GlcNAc production

To overcome the problems of GlcN degradation, and inhibitory effects of GlcN and its degradation products, GlcNAc was chosen as an alternative fermentation product. GlcNAc is stable in solution at neutral pH and the presence of 60 g l⁻¹ GlcNAc in the medium did not inhibit the growth of *E. coli* strain 7107-18 (data not shown). Moreover, GlcNAc was readily deacetylated to GlcN under relatively mild acidic conditions. More than 98% of GlcNAc (10 g l⁻¹) was converted to GlcN by treatment with 0.1 M HCl at 100 °C for 3 h.

The GlcN pathway in strain 7107-18 was altered for the production of GlcNAc by expressing a heterologous

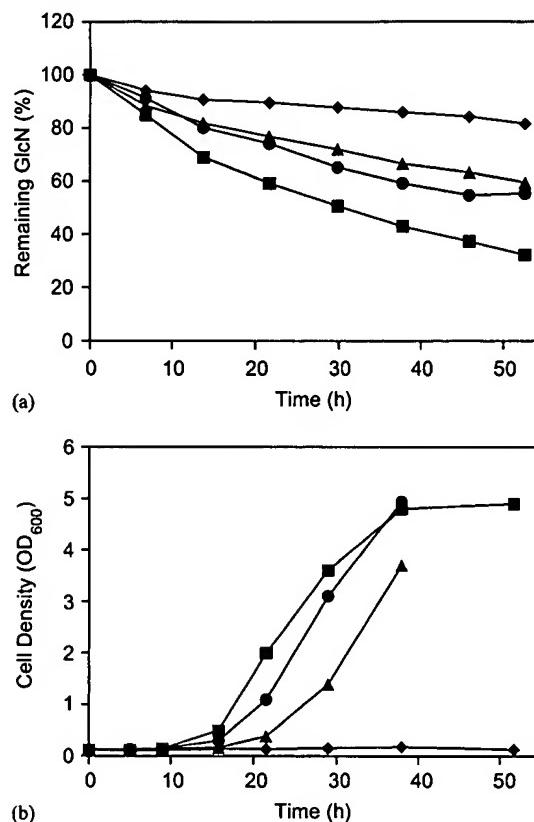


Fig. 4. GlcN degradation and inhibition of cell growth by high levels of GlcN. (a) GlcN degradation in an uninoculated growth medium at different pH: 5.5 (—◆—), 6.0 (—▲—), 6.5 (—●—) and 7.0 (—■—). The initial concentration of GlcN was 60 g l⁻¹. (b) Cell densities in cultures of *E. coli* strain 7107-18 incubated with various levels of GlcN in the medium: control (no GlcN, —■—), 10 (—●—), 20 (—▲—) and 40 g l⁻¹ (—◆—).

GNA1 gene that encodes for GlcN-6-P N-acetyltransferase (Fig. 1). Three different *GNA1* genes were expressed in *E. coli* for GlcNAc production. *GNA1* genes from *Saccharomyces cerevisiae* (*ScGNA1*), *Candida albicans* (*CaGNA1*) and *Arabidopsis thaliana* (*AtGNA1*) were cloned into the expression vector pET24d(+). Recombinant plasmids were transformed into the GlcN production *E. coli* strain 7107-18. *GNA1* transformants were grown in the M9B medium in shake flasks and induced with 0.1 mM IPTG. SDS-PAGE analysis confirmed expression of *GNA1* proteins of expected sizes. All strains exhibited comparable levels of GlmS activity (Table 2). As expected, the control strain bearing the empty vector did not show a significant level of *GNA1* activity. Expression of the yeast and higher plant *GNA1* genes led to high acetyltransferase activity in *E. coli*.

Under IPTG-induced conditions, *GNA1* transformants secreted high levels of GlcNAc into the culture medium (Table 2). Little or no GlcN (less than 0.5 g l⁻¹) could be detected in the medium. This suggests that

Table 2
GNA1 expression and its impact on cell density, acetate and GlcNAc production

| Strain | Construct ^a | Activity ^b | | OD ₆₀₀ | Acetate (g l ⁻¹) | GlcNAc (g l ⁻¹) |
|----------|---------------------------|-----------------------|------|-------------------|------------------------------|-----------------------------|
| | | GlmS | GNA1 | | | |
| 7107-88 | Vector | 0.53 | 0.1 | 3.75 | 4.4 | ND ^c |
| 7107-87 | <i>S. cerevisiae GNA1</i> | 0.48 | 19.0 | 7.80 | ND | 11.7 |
| 7107-117 | <i>C. albicans GNA1</i> | 0.36 | 22.3 | 10.00 | 0.5 | 5.1 |
| 7107-93 | <i>A. thaliana GNA1</i> | 0.32 | 6.0 | 8.70 | ND | 8.0 |

^aDifferent *GNA1* coding sequences were cloned into pET24d(+), forming *T7lac-GNA1* expression constructs. *E. coli* strains with these plasmids were grown for 23 h in M9B medium supplemented with 40 g l⁻¹ glucose, 10 g l⁻¹ ribose and 5 g l⁻¹ yeast extract. Cultures were induced with 0.2 mM IPTG at the time of inoculation.

^bEnzyme activities are expressed in $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

^cND: not detectable.

high-level expression of the acetyltransferase prevented a buildup of the intermediate GlcN-6-P. Indeed, preliminary data showed that the intracellular levels of GlcN-6-P were about 15-fold lower in the GlcNAc production strain 7107-87 than in the GlcN production strain 7107-18. Among the three *GNA1* genes tested, the *S. cerevisiae* gene outperformed the others and was selected for further strain development.

3.6. Physiology of GlcNAc production

The strain hosting the plasmid with a *T7lac-ScGNA1* cassette (7107-87) was studied under different growth conditions. In the simple mineral salt medium M9B with glucose as carbon source, the strain grew to an OD₆₀₀ of 5.4 after 24 h under non-induced conditions. However, when IPTG was added at the time of inoculation, the OD₆₀₀ at 24 h was about 30% lower. Supplementation with yeast extract improved cell growth and GlcNAc production (Table 3). Pentose sugars (ribose in particular), or gluconate also had positive effects. The effects of yeast extract were additive to those of ribose and gluconate. Addition of both ribose and yeast extract resulted in a GlcNAc level of 22.4 g l⁻¹ at 48 h in shake flasks. On the other hand, GlcN production in cultures of 7107-18 was not affected by the addition of yeast extract, pentose and gluconate (data not shown).

Strains that produced high levels of GlcNAc accumulated little or no acetate in shake flask cultures under conditions where the control strain accumulated acetate at several grams per liter (Table 2). It is important to note that the GlcNAc-producing strain grew to much higher cell densities than the control strain (Table 2) under the same growth conditions.

3.7. Optimization of GlcNAc production process

A strain bearing the *ScGNA1* plasmid (7107-87) was grown in fermentors and induced with IPTG at the time

Table 3
Effects of medium supplementations on growth and GlcNAc production in shake flask cultures

| Medium supplementation | OD ₆₀₀ | GlcNAc (g l ⁻¹) |
|---|-------------------|-----------------------------|
| Experiment I: Lactose induction | | |
| Lactose (40 g l ⁻¹) | 3.9 | 2.3 |
| Lactose (40 g l ⁻¹), yeast extract (5 g l ⁻¹) | 6.9 | 6.0 |
| Lactose (30 g l ⁻¹), glucose (10 g l ⁻¹) | 5.3 | 1.3 |
| Lactose (30 g l ⁻¹), glucose (10 g l ⁻¹), yeast extract (5 g l ⁻¹) | 12.0 | 9.0 |
| Experiment II: IPTG induction | | |
| Glucose (30 g l ⁻¹) | 3.8 | 3.0 |
| Glucose (20 g l ⁻¹), ribose (10 g l ⁻¹) | 5.7 | 6.0 |
| Glucose (30 g l ⁻¹), yeast extract (5 g l ⁻¹) | 8.7 | 9.9 |
| Glucose (20 g l ⁻¹), ribose (10 g l ⁻¹), yeast extract (5 g l ⁻¹) | 12.0 | 18.7 |
| Experiment III: IPTG induction in a medium containing glucose (30 g l⁻¹) and yeast extract (5 g l⁻¹) | | |
| Control | 10.0 | 11.1 |
| Ribose (10 g l ⁻¹) | 13.3 | 22.4 |
| Xylose (10 g l ⁻¹) | 11.0 | 12.7 |
| Arabinose (10 g l ⁻¹) | 11.5 | 15.2 |
| Potassium gluconate (10 g l ⁻¹) | 10.0 | 18.7 |

E. coli strain 7107-87 hosting a free-replicating plasmid containing a *T7lac-ScGNA1* expression cassette were grown for 48 h in the defined mineral salt medium M9B with indicated supplementations. Cultures were induced, at the time of inoculation, with IPTG (0.2 mM) or lactose as indicated.

of inoculation (early induction) or after 24 h of incubation (late induction). As shown in Fig. 5, cells grew very poorly and little GlcNAc was produced (<5 g l⁻¹) when the culture was induced early. Addition of yeast extract slightly improved growth but did not increase GlcNAc production in early-induced cultures. On the other hand, the late induction allowed good growth and high GlcNAc production (50 g l⁻¹ at 70 h). In late-induced cultures, yeast extract and ribose increased GlcNAc production by improving cell growth

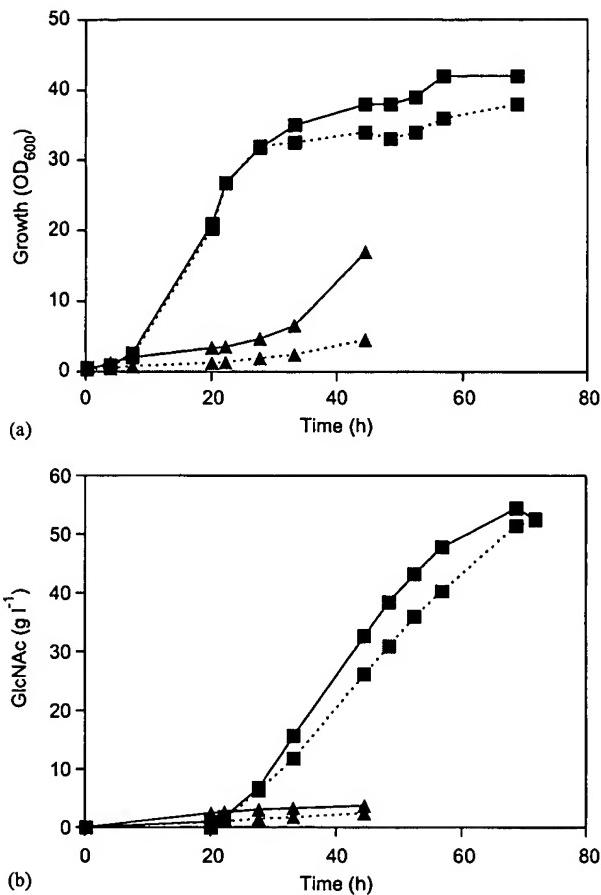


Fig. 5. Effects of the timing of IPTG induction on growth and GlcNAc production. *E. coli* strain 7107-87 (containing a *T7lac-glmS*54* expression cassette integrated in the chromosome and a *T7lac-ScGNA1* expression cassette carried on a free-replicating plasmid) was grown in one-liter fermentors. Cultures were induced with 0.4 mM IPTG at the time of inoculation (triangles) or 24 h after inoculation (squares). The medium was supplemented without (dotted lines) or with 4 g l⁻¹ yeast extract (solid lines). (a) Growth monitored by OD₆₀₀. (b) GlcNAc production.

without affecting the rate of specific formation (GlcNAc g h⁻¹ g⁻¹ dry cell weight). Further experiments showed that these supplements were not required during fermentation when cell cultures were grown initially to a high cell density before induction.

Using the strain containing the *T7lac-ScGNA1* expression plasmid, lactose induction of GlcNAc production was relatively ineffective compared to IPTG induction under some experimental conditions. Four copies of the expression cassette *T7lac-ScGNA1* were integrated into the chromosome, each at a different target site. With the integrated cassette, lactose was as efficient as IPTG in inducing GlcNAc production. Strains with one, two and three copies of integrated cassette showed 25%, 50% and 70% GNA1 activity, respectively, as compared to a plasmid-bearing strain.

GNA1 activity was not rate limiting, as levels of GlcNAc production in shake flasks and fermentors with single or multiple copies of the gene were comparable. During fermentation, GNA1 activity was elevated soon after induction and remained high throughout the 72-h run. GlmS activity was more variable, tending to decline towards the end of fermentation. Typically, high levels of GlmS activity towards the end were associated with high titers of GlcNAc.

To maximize GlcNAc production, fermentation experiments were conducted to evaluate inoculation cell density, lactose induction timing, lactose dose, glucose feeding rate, and medium composition, especially levels of phosphate, calcium and trace elements. With the improved fermentation conditions, GlcNAc production levels using strains containing two integrated copies of the *GNA1* cassette reached over 110 g l⁻¹ at 72 h (Fig. 3b). Levels of GlcN were below 1 g l⁻¹ throughout the fermentation. The process was successfully scaled up to 14- and 250-L fermentors with comparable titers. Product yield on glucose is one of the most important issues in developing a cost effective fermentation process. The maximal theoretical yield of GlcNAc on glucose is 67% by mole and 82% by weight. Due to the need for biomass production, the highest achievable yield on glucose by weight is about 70%. The weight yield on glucose in the GlcNAc fermentation described above was around 45%. With high product titers already achieved, current research is focused on maximizing the yield on glucose yield through strain and process optimization, and scaling up the fermentation process to industrial manufacturing scale.

4. Discussion

Metabolic engineering is a powerful tool that can be used to increase the capacity and efficiency of current production hosts or to create new hosts to produce novel chemicals by using advanced methods of metabolic flux analysis and molecular genetics such as gene inactivation, over-expression, deregulation, recombination and directed evolution. Metabolic engineering was successfully used in the present work to develop microbial strains for fermentation production of GlcN and GlcNAc.

E. coli was modified to accumulate GlcN by inactivating genes involved in GlcN transport and catabolism, and by over-expressing the biosynthesis gene (*glmS*). As a result, the GlcN level was increased from about 4 to 75 mg l⁻¹. GlmS is known to be sensitive to product inhibition (White, 1968; Broschat et al., 2002). GlcN-6-P strongly inhibits GlmS activity while GlcN only slightly inhibits the enzyme. Directed evolution has been increasingly used to improve enzyme characteristics (Moore and Arnold, 1996; Castle et al.,

2004; Lutz and Patrick, 2004). Mutant variants of the *E. coli* *glmS* gene were generated by error-prone PCR and clones producing GlcN at higher levels were screened by a cross-feeding plate assay. Sensitivity to production inhibition was significantly reduced in GlcN over-producing mutants. Using one of the best mutants, the level of GlcN produced in shake flask culture was 15 fold higher than the strain over-expressing the wild-type enzyme.

In general, amino sugars containing a free amino group are unstable in aqueous solution at neutral pH. GlcN has been reported to undergo spontaneous rearrangement and dimerization, forming fructosazine, D-arabinose, 5-(hydroxymethyl)-2-furaldehyde, 2,5-bis(tetrahydroxybutyl) pyrazine and other undetermined products (Kashige et al., 1995; Zhang et al., 2003). Rapid degradation of GlcN was a barrier to higher GlcN titers. In addition, GlcN was shown to inhibit cells of *E. coli* 7107-18. This is at least partially due to the deletion of the *nagB* gene. Despite the mutation in the mannose transporter operon *manXYZ*, GlcN can still be taken up via the glucose transporter, potentially leading to toxic levels of intracellular GlcN-6-P. High intracellular levels of phosphorylated sugars and amino sugars are toxic, inhibiting enzymes and causing degradation of specific mRNA species, cell lysis or stasis (White, 1968; Bernheim and Dobrogosz, 1970; Kadner et al., 1992; Park, 2001; Morita et al., 2003). Moreover, inhibitory effects of GlcN on *E. coli* 7107-18 were more pronounced in cultures where GlcN degradation products were formed before inoculation, suggesting that GlcN degradation product(s) are even more detrimental to the host cell. It was shown that GlcN degradation products, such as dehydrofructozane, cleaved plasmid DNA in vitro (Kashige et al., 1995). The mechanism(s) by which GlcN degradation products affect *E. coli* cells are not known.

To minimize GlcN degradation, alternative fermentation strategies were explored. After a growth period at a pH that was optimal for cell growth pH (6.5–7.0), pH in the fermentor was shifted to below 5.0 for GlcN production. Preliminary experiments showed only marginal improvement in GlcN titer. It would be challenging to develop an efficient *E. coli* fermentation process operating at low pH since *E. coli* generally has very low metabolic activity below pH 5.0. As an alternative, the GlcN synthesis pathway is being manipulated in yeast and lactic bacteria, which grow well at acidic pH (data not shown).

For the fermentation process using *E. coli*, another alternative was to produce a stable derivative of GlcN. GlcNAc was found to be an ideal alternative product since it is stable in solution at neutral pH and does not inhibit the growth of *E. coli* strain 7107-18. Moreover, GlcNAc was readily hydrolyzed to GlcN under relatively mild acidic conditions. Therefore, the GlcN

pathway in strain 7107-18 was altered for the production of GlcNAc. Unlike bacteria, in yeast and other eukaryotic organisms, GlcN-6-P N-acetyltransferase (encoded by the *GNA1* gene) catalyzes the formation of GlcNAc-6-P (Mio et al., 1999). Yeast and plant *GNA1* genes were expressed in *E. coli*. This led to production of GlcNAc at significant levels.

Cell growth was inhibited when *GNA1* expression was induced at the time of inoculation, which appeared to be caused by amino sugar phosphate toxicity discussed above. It has been speculated that the accumulation of amino sugar phosphates affected growth by inhibiting glucose-6-P dehydrogenase (Bernheim and Dobrogosz, 1970). This would ultimately result in a shortage of pentose phosphate, metabolites important for the cell multiplication, especially in nucleotide synthesis. After IPTG or lactose induction the intracellular levels of GlcNAc-6-P could reach a level that is inhibitory to enzyme(s) involved in pentose phosphate pathway. Indeed, supplementation with pentose sugars and gluconate improved cell growth and GlcNAc production. Yeast extract also improved growth and GlcNAc production. On the contrary, addition of pentose and yeast extract did not affect GlcN production in *E. coli* 7107-18. The inhibitory effects of GlcNAc production on host cells were effectively minimized by inducing GlcNAc production after the culture had reached a high cell density.

By optimizing fermentation medium and other process conditions, GlcNAc level in the medium reached 110 g l^{-1} . Interestingly, levels of GlcN were always below 1 g l^{-1} , indicating very efficient conversion of GlcN-6-P to GlcNAc-6-P. The latter was also efficiently dephosphorylated and secreted into the medium. It is generally accepted that phosphorylated intermediates are dephosphorylated during export from *E. coli*. The *E. coli* periplasmic space contains several phosphatases, including alkaline phosphatase and sugar phosphatases that could dephosphorylate GlcN-6-P and GlcNAc-6-P (Wanner, 1996).

Accumulation of acetate is a common obstacle to achieving high levels of recombinant protein and other fermentation products in *E. coli* (Luli and Strohl, 1990; Chen and Bailey, 1993). With excess glucose in the medium, *E. coli* cells tend to synthesize high levels of acetate and other organic acids, usually resulting in growth inhibition. Acetate production was a problem confronted in the development of the GlcN production process. GlcNAc synthesis consumes acetyl-CoA, the precursor for acetate formation. Although use of acetyl-CoA is potentially a metabolic burden on the cell, the redirection of acetyl-CoA to GlcNAc production apparently represents a significant benefit by helping to avoid acetate accumulation.

Overall, there appear to be multiple factors contributing to high GlcNAc production. GlcNAc in the medium

is inert to the host cell due to inactivation of specific transporters. *GNA1* over-expression had a dramatic impact on the pathway of amino sugar synthesis in *E. coli*. Phosphorylated amino sugar intermediates cause inhibition of growth but this was minimized by establishing a two-phase fermentation protocol. The acetylation step catalyzed by *GNA1* acted as a strong driving force to decrease intracellular GlcN-6-P levels and increase the metabolic flux to GlcNAc. The synthesis of GlcNAc also consumed acetyl-CoA, and as a consequence, minimized acetate formation.

Metabolic engineering for GlcN and GlcNAc production started with a straightforward strategy: inactivate genes involved in GlcN transport and catabolism, and over-express the biosynthesis gene (*glmS*). This strategy led to a 15-fold increase in GlcN production, but the titer remained at the milligram level. Product inhibition of *GlmS* by GlcN-6-P was identified as a critical factor in regulating the pathway. Directed evolution of the *GlmS* enzyme and over-expression of a product-resistant *GlmS* proved to be very effective in increasing GlcN production, and the titer reached multi-gram levels. However, rapid GlcN degradation and inhibitory effects of GlcN and its degradation products on the host cell prevented further titer improvement. Extending the pathway to GlcNAc, an amino sugar that is stable and inert to the *E. coli* host, by over-expressing a heterologous GlcN-6-P *N*-acetyltransferase gene (*GNA1*) represented a significant conceptual breakthrough. Finally, development and optimization of a two-phase (growth phase followed by production phase) fed batch process minimized inhibitory effects of phosphorylated amino sugars on the host cell, and resulted in the development of a high-performance fermentation process for the production of GlcNAc.

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